NUCLEOTIDE SEQUENCES FOR REGULATING GENE EXPRESSION IN PLANT TRICHOMES AND CONSTRUCTS AND METHODS UTILIZING SAME

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to nucleotide sequences for regulating gene expression in plant trichomes and, more particularly, to methods of utilizing such nucleotide sequences for synthesizing polypeptides and molecules of interest in plant trichomes.

Polypeptides can be expressed in a wide variety of cellular hosts. For economic reasons, genetically engineered unicellular microorganisms are most widely used for commercial production of polypeptides. However, in some cases, expression of mammalian proteins in unicellular organisms results in incorrect folding and processing of the expressed polypeptides leading to loss of biological or physiological activity of the obtained polypeptide. For these reasons, attempts have been made, with varying degrees of success, to express mammalian polypeptides in plants.

Transgenic plants are fast becoming a preferred system for the expression of many recombinant proteins, especially those intended for therapeutic purposes. One advantage of using plants is the potential for protein production on an agricultural scale at an extremely competitive cost. Among other advantages is that most plant transformation techniques result in a stable integration of the foreign DNA into the plant genome, so genetic recombination by crossing of transgenic plants is a simple method for introducing new genes, accumulating multiple genes into plants and avoiding the contamination of pathogens such as viruses and prions, which may affect human and animals. Furthermore, the processing and assembly of recombinant proteins in plants may also complement that in mammalian cells, which may be an advantage over the more commonly used microbial expression systems.

Although plants provide a suitable alternative to unicellular expression systems, several disadvantages characterize current approaches for production of protein in plants. First, the concentration of the produced protein is typically low (around 1% of total proteins) making purification extremely difficult. Second, other compounds may interfere with protein purification or even damage the proteins during purification. Third, expressing foreign proteins in propagated plants can lead

to environmental contamination and health risks associated with unwanted production of those proteins in cross pollinated plants.

In efforts of overcoming the above described limitations and while reducing the present invention to practice, the present inventors have discovered that plant trichomes enable compartmentalized production of foreign proteins as well as enzymatic production of novel chemicals, since many types of chemicals are naturally produced and even secreted from trichomes.

The above ground surfaces of many plants are covered with trichomes or hairs. The morphology of these structures can vary greatly with tissue type and species. Indeed, the botanical literature contains more than 300 descriptions (uniseriate, capitate-sessile, etc.) of various morphological types of such hairs (3, and references therein). Functionally, trichomes may be simple hairs that deter herbivores, guide the path of pollinators, or affect photosynthesis, leaf temperature, or water loss through increased light reflectance as in desert species. Alternatively, they may be more specialized tissues (glandular secreting trichomes) whose principal function(s) may be to produce pest- or pollinator-interactive compounds that are stored or volatilized at the plant surface. It has been suggested that in some desert species the principal role of glandular secreting trichomes is to produce such high levels of exudate that it forms a continuous layer on the plant surface. This layer may increase light reflectance and thereby reduce leaf temperature (30).

Trichomes develop projections from protodermal cells. Their structures arise from a series of anticlinal and periclinal divisions to form supporting auxiliary cells and glands. The appearance of glands atop supporting cells and the occurrence of exudate around gland cells has suggested that secretions are produced in gland cells and not by other epidermal or subepidermal cells.

In several species, such as tomato and potato, a unique type of trichomes accumulates certain protein (polyphenol oxidase) and compound (polyphenol) in the associated glands on the top of the trichome. When an insect lands on a leaf surface and contacts these trichomes, they discharge their inner compounds thereby contacting the insect and smearing it with a brown sticky compound, which is the product of enzymatic oxidation of the polyphenols (reviewed in 4).

The mass production, accumulation, and secretion of such proteins and chemicals involve a specific genetic mechanism. This genetic mechanism includes

genes (5, 6) and promoters (7, 8, 9) acting in trichome cells and cells organelles suited for accumulation and secretion of mass products. This genetic mechanism allows, for example, trichome exudates to reach 16% of total dry weight of leaves of a certain tobacco species (10) and a single protein to reach 60% of total proteins or a concentration of 14 mg/mL in the trichome content of a solanum species (11, 12). The use of this genetic mechanism was suggested for tissue specific production and accumulation of natural and heterologous proteins as well as chemicals (6). New compounds produced can be beneficial for the plant itself by increasing resistance against pests such as insects, bacteria, and fungi (6), or for Molecular Farming or Bio-Farming of human or mammalian proteins for the use as therapeutics. In the latter, harvesting the proteins produced in the trichomes can be mechanized.

Directing protein expression into trichome cells may involve the use of polynucleotides originated form different origins. A candidate source for such regulatory elements is cotton as its fiber tissue is structurally modified trichomes. The promoter sequences of cotton fiber specific genes were shown to direct *β-glucuronidase* (GUS) expression to the trichome cells of tobacco plants (7, 9). Alteration of trichomes structure or chemistry by, for example, increasing cotton trichome length or by producing pigments in the fiber could be beneficial for the cotton industry.

Natural chemicals of trichomes are already used as flavor, aroma, medicinals, pesticides, and cosmetic ingredients (13, 14). Natural chemicals content was altered using antisense and co-suppression methods (6). However, enzymatic modifications of trichomes compounds via genetic engineering of genes, designed to produce other useful compounds in trichomes, was never shown before.

Several limitations had narrowed so far the use of plant trichomes for commercially production of heterologous proteins and novel chemicals.

First, protein yield is very limited in trichome cells and to date there is no existing method that enables commercially significant production of proteins in these cells. Although there are known promoter sequences that are capable of directing protein synthesis in trichomes (7, 8, 9), proteins expressed therefrom accumulated at average levels of accumulation of a single trichome protein and thus these promoters cannot be considered commercially useful for protein production, as is. Second, trichomes usually produce a mix of several metabolites, some of which (e.g., phenols

and alkaloids), can inhibit protein accumulation or substantially hinder purification of desired compounds produced in trichomes (See material and methods in 12). Thus, reducing the levels of such harmful metabolites is required in order to improve harvesting and collection of the desired products. Third, the production of novel compounds in plants always involves risks of escape of genetic material (pollen and seeds) to the environment with potential damage to other organisms (plants, insects animals, human). Hence, when producing novel compounds one should consider the elimination of the possible spread of the new genetic material.

There is thus a widely recognized need for and it would be highly advantageous to have nucleotide sequences for regulating gene expression in plant trichomes methods of utilizing such nucleotide sequences for generating molecules of interest in plant trichomes.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided an isolated polynucleotide comprising a nucleic acid sequence at least 80 % identical to SEQ ID NO: 23, 26 or 29, wherein the nucleic acid sequence is capable of regulating expression of at least one polynucleotide sequence operably linked thereto in trichomes.

According to another aspect of the present invention there is provided a nucleic acid construct comprising the isolated polynucleotide.

According to further features in preferred embodiments of the invention described below, the nucleic acid construct further comprising at least one heterologous polynucleotide operably linked to the isolated polynucleotide.

According to still further features in the described preferred embodiments the nucleic acid construct further comprises, a nucleic acid sequence encoding a peptide capable of directing transport of a polypeptide fused thereto into a subcellular compartment of a trichome.

According to still further features in the described preferred embodiments the nucleic acid sequence is selected from the group consisting of SEQ ID NOs: 59, 61, 63, 65 and 67.

According to yet another aspect of the present invention there is provided a transgenic cell comprising the nucleic acid construct.

According to still another aspect of the present invention there is provided a transgenic plant comprising the nucleic acid construct.

According to an additional aspect of the present invention there is provided an isolated polynucleotide comprising a nucleic acid sequence encoding a peptide capable of directing transport of a polypeptide fused thereto into a subcellular compartment of a trichome, wherein the peptide is encoded by the polynucleotide sequence set forth in SEQ ID NO: SEQ ID NOs: 59, 61, 63, 65 and 67.

According to yet an additional aspect of the present invention there is provided a nucleic acid construct comprising the isolated polynucleotide.

According to still further features in the described preferred embodiments the nucleic acid construct further comprising an expressible polynucleotide sequence translationally fused to the nucleic acid sequence encoding the peptide.

According to still an additional aspect of the present invention there is provided a method of producing a polypeptide of interest in plant trichomes, the method comprising:

(a) expressing the polypeptide of interest in the plant trichomes; and

(b) down-regulating a level of at least one molecule endogenous to the plant trichomes, the at least one molecule being capable of interfering with expression, accumulation or stability of the polypeptide of interest.

According to still further features in the described preferred embodiments step (b) is effected by gene silencing.

According to a further aspect of the present invention there is provided a method of producing a molecule of interest in plant trichomes, the method comprising: (a) expressing a polypeptide capable of directly or indirectly increasing a level of the molecule of interest in the plant trichomes; and (b) down-regulating a level of at least one molecule endogenous to the plant trichomes, the at least one molecule being capable of interfering with accumulation or stability of the molecule of interest, thereby producing the molecule in the plant trichomes.

According to still further features in the described preferred embodiments the polypeptide is endogenously expressed in the plant trichomes.

According to still further features in the described preferred embodiments the expressing the polypeptide in the plant trichomes is effected by introducing into the

plant trichomes a nucleic acid sequence encoding the polypeptide positioned under a transcriptional control of a promoter functional in the plant trichomes.

According to still further features in the described preferred embodiments the promoter is as set forth in SEQ ID NO: 23, 26, 29, 35, 38, 39, 42, 48, 50 or 51.

According to still further features in the described preferred embodiments the nucleic acid sequence encoding the polypeptide of interest further encodes a peptide capable of directing transport of the polypeptide fused thereto into a subcellular compartment of the plant trichomes.

According to still further features in the described preferred embodiments the at least one molecule is an enzyme or a metabolite.

According to still further features in the described preferred embodiments the metabolite is selected from the group consisting of polyphenols, ketones, terpenoids, phenylpropanoids and alkaloids.

According to still further features in the described preferred embodiments the enzyme is PPO.

According to still further features in the described preferred embodiments step (b) is effected by gene silencing.

According to yet a further aspect of the present invention there is provided a plant genetically modified to express a molecule of interest in trichomes, wherein the plant is further modified or selected capable of accumulating less than 50 % of average volume of undesired compounds in trichome cells of the plant species.

According to still further features in the described preferred embodiments at least a portion of cells of the plant are genetically modified to include an expression construct including a polynucleotide sequence of a trichome specific promoter.

According to still further features in the described preferred embodiments the expression construct further includes an additional polynucleotide sequence encoding a peptide capable of directing transport of a polypeptide fused thereto into a subcellular compartment of the trichome, whereas the additional polynucleotide is translationally fused to the polynucleotide sequence.

According to still further features in the described preferred embodiments at least a portion of cells of the plant are genetically modified to include an expression construct including a first polynucleotide sequence encoding the polypeptide

translationally fused to a second polynucleotide sequence encoding a peptide capable of directing transport of a polypeptide fused thereto into a trichome.

According to still further features in the described preferred embodiments the expression or accumulation is in a subcellular compartment of trichomes.

According to still further features in the described preferred embodiments the subcellular compartment is a leucoplast.

According to still further features in the described preferred embodiments the trichome specific promoter is set forth by SEQ ID NO: 23, 26 or 29.

According to still further features in the described preferred embodiments the trichome specific promoter is set forth by SEQ ID NO: 23, 26, 29, 35, 38, 39, 42 or 45.

According to still further features in the described preferred embodiments the additional polynucleotide sequence is set forth by SEQ ID NO: 59, 61, 63, 65 or 67.

40. The plant of claim 31, wherein the molecule of interest is not a reporter polypeptide.

According to still further features in the described preferred embodiments the plant is modified or selected capable of generating a trichome density above 50,000 trichomes/gr leaf tissue.

According to still further features in the described preferred embodiments the plant is modified or selected capable of generating a trichome size of 50 % above average size of the plant species.

According to still further features in the described preferred embodiments the plant is modified or selected capable of generating leaf surface size at least 25 % above average size of the plant species.

According to still further features in the described preferred embodiments the plant is modified or selected capable of generating total leaf number at least 50 % above average leaf number of the plant species.

According to still further features in the described preferred embodiments the plant is sterile.

According to still further features in the described preferred embodiments the plant is further genetically modified capable of secreting the exogenous polypeptide from trichome cells.

According to still a further aspect of the present invention there is provided a method of harvesting trichomes and/or exudates and/or content thereof, the method comprising: (a) incubating a trichome-containing plant tissue in a liquid such that trichome exudates and content is released into the liquid, wherein incubating is effected while avoiding friction of the trichome-containing plant tissue with a solid phase; and (b) collecting the liquid, to thereby harvest the trichome exudates and content.

According to still further features in the described preferred embodiments the liquid includes an antioxidant.

According to still further features in the described preferred embodiments the antioxidant is selected from the group consisting of citric acid, ascorbic acid and sodium bisulfite

According to still further features in the described preferred embodiments the liquid is water.

According to still further features in the described preferred embodiments the trichome-containing plant tissue is selected from the group consisting of a shoot, a flower and a leaf.

According to still a further aspect of the present invention there is provided an apparatus for mechanical harvesting of trichome exudates and content, the apparatus comprising a mechanism designed and configured for mechanically aggitating a trichome-containing plant tissue in a fluid and collecting the fluid to containing thetrichome exudates or content..

The present invention successfully addresses the shortcomings of the presently known configurations by providing nucleotide sequence for regulating gene expression in plant trichomes and methods of utilizing such nucleotide sequences for generating molecules in plant trichomes

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIG. 1a is a prior art schematic illustration of various tomato trichomes. Type VI glandular trichomes naturally accumulate high levels of the PPO enzyme (Luckwill LC. 1943. The Aberden University Press, Aberden, Scotland).

FIGs. 1b-h are photomicrographs depicting trichome specific expression of GUS under the regulation of the CaMV 35S, TR2, TR5, TR11, TR25 or TR27P promoters. Figure 1b - Trichomes of wild-type tomato plants. Figure 1c - Trichomes of tomato plants overexpressing GUS under the constitutive CaMV 35S promoter. Figures 1b-h - Trichomes of tomato plants overexpressing GUS with different trichome promoters

FIG. 2 is a photograph depicting total protein yield of chemically extracted trichome cells as determined by coomassie staining.

FIG. 3 is a photograph depicting decreased PPO activity in the presence of increasing concentrations of sodium bisulfite, as indicated by medium browning, which is indicative of PPO activity.

FIGs. 4a-c schematically illustrate various embodiments of a trichome mechanical harvester constructed in accordance with the teachings of the present invention.

FIGs. 5a-c are graphs showing the effect of pruning on leaf number of 3 tomato cultivars. Figure 5a cultivar 678; Figure 5b cultivar 1312; Figure 5c cultivar 2545. Treatment 1 - Plant shoot number was not limited, plant height was limited to 1 m, flowers were cut-off before fruit set; Treatment 2 - plant shoot number was not

limited, plant height was limited to 2 m, flowers were cut-off before fruit set; Treatment 3 - plant shoot number was not limited, leading apical meristem was cut (i.e. breaking apical dominance) when reached 0.5m, flowers were cut-off before fruit set. Treatment 4 – control plants were treated for tomato fruit set, such that each plant includes 2 shoots. Flowers and fruits were untouched.

FIGs. 6a-d are graphs depicting expression levels of three trichome-expressed genes as determined by RT-PCR. Expression is shown as fold increase over house-keeping gene expression. 273_1 is *L.hirsutum* var glabratum cultivar; 247 is *L. esculentum* cultivar. Tissue key: L- Leaves; L-T- Leaves minus Trichomes; T1 and T2 are two independent RNA samples of Trichome cells.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is nucleotide sequence for regulating gene expression in plant trichomes which can be utilized for generating molecules in plant trichomes. Specifically the present invention is of plants which are modified for enhanced expression and accumulation of molecules in plant trichomes.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Trichomes are hairy-like epidermal multi-cell structures found on the outer surface of leaves, stems and flowers of about 20-30 % of plant species. Their Main function is associated with plant protection against insects, microbes and herbivores due to their ability to massively accumulate and secrete pest-deterrent phytochemicals. Other functions include water absorption, seed dispersal and abrasion protection.

The high production capacity of trichomes prompted their utilization as "green-factories" for producing commercially useful compounds (e.g., U.S. Pat. No. 6,730,826). However, mass production of recombinant proteins in trichomes is limited

by poor production efficiency and the presence of metabolites and enzymes, which may interfere with purification and activity of the desired compounds.

While reducing the present invention to practice the present inventors have devised a novel approach for increasing expression, accumulation and harvesting of molecules in plant trichomes, while reducing the chances of accidentaly spreading the non-plant genetic material used to generate the molecules.

As is illustrated in the Examples section, the present inventors have uncovered that by reducing the concentration of undesired compounds in trichome exudates an enhanced level of expression, accumulation and/or purification of commercially valuable molecules within the trichomes can be achieved. Furthermore the present inventors uncovered through laborious experimentation and time consuming analysis a number of novel trichome active regulatory elements (see Example 1), which enable protein over-expression in trichomes (see Examples 2-4).

These findings allow, for the first time, improved molecular farming in trichome cells.

Thus, according to one aspect of the present invention there is provided a method of producing a molecule of interest in plant trichomes.

As used herein the term "trichome" refers to both a "simple" (also termed "non-glandular") trichome and a "glandular-secreting" (GST) trichome. Preferably, the term trichome refers to a GST trichome.

As used herein the term "molecule" refers to at least one small molecule chemical (e.g., nicotine, flavomoids). Such molecules can be naturally expressed or present in trichomes or can be direct or indirect expression products of heterologous polynucleotides. Examples of molecules which can be produced in trichome cells according to this aspect of the present invention include, but are not limited to, oils, dyes, flavors, biofuels, or industrial biopolymers, pharmaceuticals, nutraceuticals and cosmeceuticals.

As used herein the term "producing" refers to the process of expressing and/or accumulating the molecule in trichome cells. When appropriate, producing may also refer to subsequent steps of purifying the molecule from the trichome cells.

The method, according to this aspect of the present invention, is effected by upregulating expression of a polypeptide capable of directly or indirectly increasing a level of the molecule of interest; and down-regulating a level of at least one molecule

endogenous to the plant trichome, which is capable of interfering with the production of the molecule in the plant trichomes, thereby producing the molecule in the plant trichomes.

Examples of polypeptides capable of directly or indirectly increasing the level of the molecule of interest include include for example, trichome specific transcription factors which promote expression in trichome cells. Alternatively, the polypeptide can be an enzyme participating in a biochemical pathway, which produces the molecule in the trichome.

Expression of polypeptides in plant trichomes according to this aspect of the present invention, may be effected by placing a polynucleotide encoding the polypeptide of interest under the regulation of a cis-acting regulatory element capable of directing expression from the polynucleotide in trichome cells.

As used herein, the phrase "cis acting regulatory element" refers to a polynucleotide sequence, preferably a promoter, which binds a trans acting regulator and regulates the transcription of a coding sequence located downstream thereto in trichome cells.

It will be appreciated that a regulatory sequence is "operably linked" to a coding polynucleotide sequence if it is capable of exerting a regulatory effect on the coding sequence linked thereto. Preferably, the regulatory sequence is positioned 1-500 bp upstream of the ATG codon of the coding nucleic acid sequence, although it will be appreciated that regulatory sequences can also exert their effect when positioned elsewhere with respect to the coding nucleic acid sequence (e.g., within an intron).

A number of trichome active promoters are known in the art which can be used in accordance with the present invention. Examples include, but are not limited to, the CYP71D16 trichome-specific promoter [Wang E. J Exp Bot. (2002) 53(376):1891-7, see U.S. Pat. No. 6,730,826] and the cotton LTP3 and LTP6 promoters (7,9).

Methods of identifying trichome active or specific promoters are well described in Examples 1-3 of the Examples section which follows.

As mentioned hereinabove, the present inventors have identified a number of cis-acting regulatory elements, which are capable of regulating transcription of coding nucleic acid sequences operably linked thereto in trichome cells.

Thus the present invention provides an isolated polynucleotide having a nucleic acid sequence at least 80 % identical to SEQ ID NO: 23, 26 or 29, wherein the nucleic acid sequence is capable of regulating expression of at least one polynucleotide sequence operably linked thereto in trichomes.

According to other embodiments of this aspect of the present invention the nucleic acid sequence of the present invention is at least 80 % identical to SEQ ID NO: 35, 38, 39, 42, 45, 48, 51 or 54.

Preferably, the polynucleotides (promoters) of the present invention are modified to create variations in the molecule sequences such as to enhance their promoting activities, using methods known in the art, such as PCR-based DNA modification, or standard DNA mutagenesis techniques, or by chemically synthesizing the modified polynucleotides.

Accordingly, the sequences set forth in SEQ ID NOs: 23, 26, 29, 35, 38, 39, 42, 45, 48, 51 and 54 may be truncated or deleted and still retain the capacity of directing the transcription of an operably linked DNA sequence in trichomes. The minimal length of a promoter region can be determined by systematically removing sequences from the 5' and 3'-ends of the isolated polynucleotide by standard techniques known in the art, including but not limited to removal of restriction enzyme fragments or digestion with nucleases.

In another approach, novel hybrid promoters can be designed or engineered by a number of methods. Many promoters contain upstream sequences which activate, enhance or define the strength and/or specificity of the promoter, such as described, for example, by Atchison [Ann. Rev. Cell Biol. 4:127 (1988)]. T-DNA genes, for example contain "TATA" boxes defining the site of transcription initiation and other upstream elements located upstream of the transcription initiation site modulate transcription levels [Gelvin In: Transgenic Plants (Kung, S.-D. and Us,R., eds, San Diego: Academic Press, pp.49-87, (1988)]. Another chimeric promoter combined a trimer of the octopine synthase (ocs) activator to the mannopine synthase (mas) activator plus promoter and reported an increase in expression of a reporter gene [Min Ni *et al.*, The Plant Journal 7:661 (1995)]. The upstream regulatory sequences of the present invention can be used for the construction of such chimeric or hybrid promoters. Methods for construction of variant promoters include, but are not limited to, combining control elements of different promoters or duplicating portions or

regions of a promoter (see for example, U.S. Pat. Nos. 5,110,732 and 5,097,025). Those of skill in the art are familiar with the specific conditions and procedures for the construction, manipulation and isolation of macromolecules (e.g., DNA molecules, plasmids, etc.), generation of recombinant organisms and the screening and isolation of genes, [see for example Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, (1989); Mailga *et al.*, Methods in Plant Molecular Biology, Cold Spring Harbor Press, (1995); Birren *et al.*, Genome Analysis: volume 1, Analyzing DNA, (1997); volume 2, Detecting Genes, (1998); volume 3, Cloning Systems, (1999); and volume 4, Mapping Genomes, (1999), Cold Spring Harbor, N.Y].

The above-described nucleic acid sequences (promoters) can be used to drive expression of a heterologous polynucleotide of interest in trichome cells. Preferably, the heterologous polynucleotide can encode any naturally occurring or man-made recombinant protein, such as pharmaceutical proteins [e.g., growth factors and antibodies Schillberg Naturwissenschaften. (2003) Apr;90(4):145-55] and food additives. It will be appreciated that molecular farming is a well-proven way of producing a range of recombinant proteins, as described in details in Ma Nat Rev Genet. 2003 Oct;4(10):794-805; Twyman Trends Biotechnol. 2003 Dec;21(12):570-8.

To facilitate accumulation of the polypeptide of interest in trichome cells, it may be beneficial to translationally link the heterologous polynucleotide encoding the polypeptide to a signal peptide-encoding sequence which is capabale of directing transport of the polypeptide into sub-cellular organelle of a the trichome. Examples of subcellular organelles of trichome cells include, but are not limited to, leucoplasts, chloroplasts, chromoplasts, mitochondria, nuclei, peroxisomes, endoplasmic reticulum and vacuoles. Preferably the signal peptide of this aspect of the present invention is a leucoplast localization signal. It is appreciated that since the protein is not accumulated in the cytoplasm, but rather in the subcellular organelle of the trichomes, it is expected to be stored in relatively high concentrations without being exposed to the degrading compounds present in the trichome vacuole. Examples of signal peptides which may be used in accordance with the present invention include, but are not limited to, the stroma or lumen directing signal peptides of PPOA and PPOD (SEQ ID NO: 60, 62, 64, 66 and 76, see Example 3). Polynucleotides encoding these signal peptides are set forth in SEQ ID NOs: 59, 61, 63, 65 and 75.

The polynucleotides (i.e., trichome active promoter sequence, signal peptide encoding polynucleotide) of the present invention, or fragments, variants or derivatives thereof, can be incorporated into nucleic acid constructs, preferably expression constructs (i.e., expression vectors), which can be introduced and replicate in a plant cell, such as a trichome. Such nucleic acid constructs may include the heterologous polyncueotide of interest such as described hereinabove, operably liked to any of the promoter sequences of the present invention.

The nucleic acid construct can be, for example, a plasmid, a bacmid, a phagemid, a cosmid, a phage, a virus or an artificial chromosome. Preferably, the nucleic acid construct of the present invention is a plasmid vector, more preferably a binary vector.

The phrase "binary vector" refers to an expression vector which carries a modified T-region from Ti plasmid, allowing multiplication both in *E. coli* and in *Agrobacterium* cells, and usually comprising selection gene(s). Such a binary vector suitable for the present invention is described in Example 1 of the Examples section which follows.

The nucleic acid construct of the present invention can be utilized to transform a host cell. Preferably a plant cell. Preferably, the nucleic acid construct of the present invention is used to transform at least a portion of cells of a plant.'

Methods of introducing nucleic acid constructs into a cell or a plant are well known in the art. Accordingly, suitable methods for introducing nucleic acid sequences into plants include, but are not limited to, bacterial infection, direct delivery of DNA (e.g., via PEG-mediated transformation, desiccation/inhibition-mediated DNA uptake, electroporation, agitation with silicon carbide fibers, and acceleration of DNA coated particles, such as described by Potrykus Ann. Rev. Plant Physiol. Plant Mol. Biol. 42:205-225 (1991).

Methods for specifically transforming dicots primarily use *Agrobacterium tumefaciens*. For example, transgenic plants reported include but are not limited to cotton (U.S. Pat. Nos. 5,004,863, 5,159,135, 5,518,908; and WO 97/43430), soybean [U.S. Pat. Nos. 5,569,834, 5,416,011; McCabe *et al.*, Bio/Technology, 6:923 (1988); and Christou *et al.*, Plant Physiol., 87:671, (1988)]; Brassica (U.S. Pat. No. 5,463,174), and peanut [Cheng *et al.*, Plant Cell Rep., 15: 653, (1996)]. Similar methods have been reported in the transformation of monocots. Transformation and

plant regeneration using these methods have been described for a number of crops including but not limited to asparagus [*Asparagus officinalis*; Bytebier *et al.*, Proc. Natl. Acad. Sci. U.S.A., 84: 5345, (1987); barley *(Hordeum vulgarae*; Wan and Lemaux, Plant Physiol., 104: 37, (1994)]; maize [*Zea mays*; Rhodes, C. A., *et al.*, Science, 240: 204, (1988); Gordon-Kamm, *et al.*, Plant Cell, 2: 603, (1990); Fromm, *et al.*, Bio/Technology, 8: 833, (1990); Koziel, *et al.*, Bio/Technology, 11: 194, (1993)]; oats [*Avena sativa*; Somers, *et al.*, Bio/Technology, 10: 1589, (1992)]; orchardgrass [*Dactylis glomerata*; Horn, *et al.*, Plant Cell Rep., 7: 469, (1988); rice [*Oryza sativa*, including indica and japonica varieties, Toriyama, *et al.*, Bio/Technology, 6: 10, (1988); Zhang, *et al.*, Plant Cell Rep., 7: 379, (1988); Luo and Wu, Plant Mol. Biol. Rep., 6: 165, (1988); Zhang and Wu, Theor. Appl. Genet., 76: 835, (1988); Christou, *et al.*, Bio/Technology, 9: 957, (1991); sorghum [*Sorghum bicolor*; Casas, A. M., *et al.*, Proc. Natl. Acad. Sci. U.S.A., 90: 11212, (1993)]; sugar cane [*Saccharum* spp.; Bower and Birch, Plant J., 2: 409, (1992)]; tall fescue [*Festuca arundinacea*; Wang, Z. Y. *et al.*, Bio/Technology, 10: 691, (1992)]; turfgrass [*Agrostis palustris*; Zhong *et al.*, Plant Cell Rep., 13: 1, (1993)]; wheat [*Triticum aestivum*; Vasil *et al.*, Bio/Technology, 10: 667, (1992); Weeks T., *et al.*, Plant Physiol., 102: 1077, (1993); Becker, *et al.*, Plant, J. 5: 299, (1994)], and alfalfa [Masoud, S. A., *et al.*, Transgen. Res., 5: 313, (1996)]. It is apparent to those of skill in the art that a number of transformation methodologies can be used and modified for production of stable transgenic plants from any number of target crops of interest.

The transformed plants can be analyzed for the expression features conferred by the polynucleotides of the present invention, using methods known in the art for the analysis of transformed plants (see Example 4 of the Examples section which follows). A variety of methods are used to assess gene expression and determine if the introduced gene(s) is integrated, functioning properly, and inherited as expected. Preferably, the promoters are evaluated by determining the expression levels and the expression features of genes to which the promoters are operatively linked. A preliminary assessment of promoter function can be determined by a transient assay method using reporter genes, but a more definitive promoter assessment can be determined from the analysis of stable plants. Methods for plant analysis include but are not limited to Southern blots or northern blots, PCR-based approaches, biochemical analyses, phenotypic screening methods, field evaluations, and

immunodiagnostic assays. These methods may also be used to assess gene silencing, which is described hereinbelow.

As mentioned hereinabove, to enhance expression and/or accumulation of the molecule of interest in trichome cells and/or to facilitate purification of the molecule from trichome cells, down-regulation of at least one molecule endogenous to the plant trichomes and interfering with these processes is effected.

Trichomes are known to include a number of compounds (e.g., metabolites), which interfere with the production of molecules in these specialized cells. These metabolites include, for example polyphenols, ketones, terpenoids (e.g., monoterpenes, sesquiterpenes, diterpenes and triterpenes), mixed terpenes, phenylpropanoids and alkaloids. Other trichome components which may be preferably reduced to improve, expression, accumulation and purification of the molecules of this aspect of the present invention include proteases, and PPO (see Example 5 of the Examples section). For example downregulation PPO in trichome plastids would allow the recruitment of the protein translation machinery to a novel peptide and also to increase storage space in trichome plastids. Another example is reducing enzymatic activity of the polyphenols biosynthetic pathway to thereby decrease/eliminate the production of polyphenols which make it difficult to harvest and purify proteins from trichomes (see above). Such enzymes include, but are not limited to, Phenylalanine ammonia-lyase (PAL, Acc. No. M90692, M83314), Cinnamate-4-hydroxylase (CA4H, GenBank Accession No. Z70216, AI490789), 4-Coumarate:coenzyme A ligase (4CL, GenBank Accession Nos. AW034240, AF211800), chalcone and stilbene synthase (CHS, Acc. No. GenBank Accession No. X55195), Chalcone isomerase (CHI, Acc. No. GenBank Accession No. AY348871), F3H, flavanone 3-hydroxylase - naringenin 3-dioxygenase (F3OH), flavanone 3-hydroxylase - naringenin 3-dioxygenase (FDR), dihydroflavonol-4-reductase (DFR, GenBank Accession No. Z18277).

Down-regulation of such trichome components may be effected by down-regulating genes which are involved in the production or accumulation of these components. For example, gene products which are involved in exudate synthesis may be revealed by genome and EST mining and directed gene knock-out. Gene mining includes the identification in public databases (e.g., GenBank www.ncbi.nlm.nih.gov/Genbank/index/html) of ortholohgous sequences deriving from the plant of interest which share homology with known genes in the pathway using

sequence alignment software such as BLAST (www.ncbi.nlm.nih.gov/BLAST). Alternatively, trichome EST libraries may be useful for identifying genes which are involved in metabolite synthesis [see for example Lange (2000) Proc. Natl. Acad. Sci. 97:2934-2939; Gang (2001) Plant Physiology 125:539-555].

Once genes associated directly or indirectly with metabolite synthesis are identified, they are down-regulated either at the nucleic acid level and/or at the protein level (e.g., antibodies).

An agent capable of downregulating gene expression is a small interfering RNA (siRNA) molecule. RNA interference is a two step process. the first step, which is termed as the initiation step, input dsRNA is digested into 21-23 nucleotide (nt) small interfering RNAs (siRNA), probably by the action of Dicer, a member of the RNase III family of dsRNA-specific ribonucleases, which processes (cleaves) dsRNA (introduced directly or via a transgene or a virus) in an ATP-dependent manner. Successive cleavage events degrade the RNA to 19-21 bp duplexes (siRNA), each with 2-nucleotide 3' overhangs [Hutvagner and Zamore Curr. Opin. Genetics and Development 12:225-232 (2002); and Bernstein Nature 409:363-366 (2001)].

In the effector step, the siRNA duplexes bind to a nuclease complex to from the RNA-induced silencing complex (RISC). An ATP-dependent unwinding of the siRNA duplex is required for activation of the RISC. The active RISC then targets the homologous transcript by base pairing interactions and cleaves the mRNA into 12 nucleotide fragments from the 3' terminus of the siRNA [Hutvagner and Zamore Curr. Opin. Genetics and Development 12:225-232 (2002); Hammond *et al.* (2001) Nat. Rev. Gen. 2:110-119 (2001); and Sharp Genes. Dev. 15:485-90 (2001)]. Although the mechanism of cleavage is still to be elucidated, research indicates that each RISC contains a single siRNA and an RNase [Hutvagner and Zamore Curr. Opin. Genetics and Development 12:225-232 (2002)].

Because of the remarkable potency of RNAi, an amplification step within the RNAi pathway has been suggested. Amplification could occur by copying of the input dsRNAs which would generate more siRNAs, or by replication of the siRNAs formed. Alternatively or additionally, amplification could be effected by multiple turnover events of the RISC [Hammond *et al.* Nat. Rev. Gen. 2:110-119 (2001), Sharp Genes. Dev. 15:485-90 (2001); Hutvagner and Zamore Curr. Opin. Genetics and Development 12:225-232 (2002)]. For more information on RNAi see the following

reviews Tuschl ChemBiochem. 2:239-245 (2001); Cullen Nat. Immunol. 3:597-599 (2002); and Brantl Biochem. Biophys. Act. 1575:15-25 (2002).

Synthesis of RNAi molecules suitable for use with the present invention can be effected as follows. First, the mRNA target sequence is scanned downstream of the AUG start codon for AA dinucleotide sequences. Occurrence of each AA and the 3' adjacent 19 nucleotides is recorded as potential siRNA target sites. Preferably, siRNA target sites are selected from the open reading frame, as untranslated regions (UTRs) are richer in regulatory protein binding sites. UTR-binding proteins and/or translation initiation complexes may interfere with binding of the siRNA endonuclease complex [Tuschl ChemBiochem. 2:239-245]. It will be appreciated though, that siRNAs directed at untranslated regions may also be effective, as demonstrated for GAPDH wherein siRNA directed at the 5' UTR mediated about 90 % decrease in cellular GAPDH mRNA and completely abolished protein level (www.ambion.com/techlib/tn/91/912.html).

Second, potential target sites are compared to an appropriate genomic database (e.g., human, mouse, rat etc.) using any sequence alignment software, such as the BLAST software available from the NCBI server (www.ncbi.nlm.nih.gov/BLAST/). Putative target sites which exhibit significant homology to other coding sequences are filtered out.

Qualifying target sequences are selected as template for siRNA synthesis. Preferred sequences are those including low G/C content as these have proven to be more effective in mediating gene silencing as compared to those with G/C content higher than 55 %. Several target sites are preferably selected along the length of the target gene for evaluation. For better evaluation of the selected siRNAs, a negative control is preferably used in conjunction. Negative control siRNA preferably include the same nucleotide composition as the siRNAs but lack significant homology to the genome. Thus, a scrambled nucleotide sequence of the siRNA is preferably used, provided it does not display any significant homology to any other gene.

Antisense and siRNA technology has been used in selective downregulation of two tobacco trichome genes encoding different enzymes [Wang (2002); J. of Exp. Bot. 53:1891-1897; Wang (2003) Planta 216:686-691]. siRNA oligonucleotides for downregulating PPO for example, may be generated by inserting the cDNA sequence of PPO (GenBank Accession No: Z12833 for PPOA, GenBank Accession No.

Z12836 for PPOD) to an siRNA selection software such as provided by www.ambion.com.

Another agent capable of downregulating gene expression is a DNAzyme molecule capable of specifically cleaving an mRNA transcript or DNA sequence of the target. DNAzymes are single-stranded polynucleotides which are capable of cleaving both single and double stranded target sequences (Breaker, R.R. and Joyce, G. Chemistry and Biology 1995;2:655; Santoro, S.W. & Joyce, G.F. Proc. Natl, Acad. Sci. USA 1997;943:4262) A general model (the "10-23" model) for the DNAzyme has been proposed. "10-23" DNAzymes have a catalytic domain of 15 deoxyribonucleotides, flanked by two substrate-recognition domains of seven to nine deoxyribonucleotides each. This type of DNAzyme can effectively cleave its substrate RNA at purine:pyrimidine junctions (Santoro, S.W. & Joyce, G.F. Proc. Natl, Acad. Sci. USA 199; for rev of DNAzymes see Khachigian, LM [Curr Opin Mol Ther 4:119-21 (2002)].

Examples of construction and amplification of synthetic, engineered DNAzymes recognizing single and double-stranded target cleavage sites have been disclosed in U.S. Pat. No. 6,326,174 to Joyce *et al.*

Downregulation of gene expression can also be effected by using an antisense polynucleotide capable of specifically hybridizing with an mRNA transcript encoding the target polypeptide of interest.

Design of antisense molecules which can be used to efficiently and specifically downregulate gene expression must be effected while considering two aspects important to the antisense approach. The first aspect is delivery of the oligonucleotide into the cytoplasm of the appropriate cells, while the second aspect is design of an oligonucleotide which specifically binds the designated mRNA within cells in a way which inhibits translation thereof.

In addition, algorithms for identifying those sequences with the highest predicted binding affinity for their target mRNA based on a thermodynamic cycle that accounts for the energetics of structural alterations in both the target mRNA and the oligonucleotide are also available [see, for example, Walton *et al.* Biotechnol Bioeng 65: 1-9 (1999)].

Such algorithms have been successfully used to implement an antisense approach in cells. For example, the algorithm developed by Walton et al. enabled

scientists to successfully design antisense oligonucleotides for rabbit beta-globin (RBG) and mouse tumor necrosis factor-alpha (TNF alpha) transcripts. The same research group has more recently reported that the antisense activity of rationally selected oligonucleotides against three model target mRNAs (human lactate dehydrogenase A and B and rat gp130) in cell culture as evaluated by a kinetic PCR technique proved effective in almost all cases, including tests against three different targets in two cell types with phosphodiester and phosphorothioate oligonucleotide chemistries.

In addition, several approaches for designing and predicting efficiency of specific oligonucleotides using an *in vitro* system were also published (Matveeva *et al.*, Nature Biotechnology 16: 1374 - 1375 (1998)].

Another agent capable of downregulating gene expression is a ribozyme molecule capable of specifically cleaving an mRNA transcript encoding the target polypeptide. Ribozymes are being increasingly used for the sequence-specific inhibition of gene expression by the cleavage of mRNAs encoding proteins of interest [Welch *et al.*, Curr Opin Biotechnol. 9:486-96 (1998)]. The possibility of designing ribozymes to cleave any specific target RNA has rendered them valuable tools in both basic research and therapeutic applications.

An additional method of regulating the expression of a gene in cells is via triplex forming oligonuclotides (TFOs). Recent studies have shown that TFOs can be designed which can recognize and bind to polypurine/polypirimidine regions in double-stranded helical DNA in a sequence-specific manner. These recognition rules are outlined by Maher III, L. J., et al., Science,1989;245:725-730; Moser, H. E., et al., Science,1987;238:645-630; Beal, P. A., et al, Science,1992;251:1360-1363; Cooney, M., et al., Science,1988;241:456-459; and Hogan, M. E., et al., EP Publication 375408. Modification of the oligonuclotides, such as the introduction of intercalators and backbone substitutions, and optimization of binding conditions (pH and cation concentration) have aided in overcoming inherent obstacles to TFO activity such as charge repulsion and instability, and it was recently shown that synthetic oligonucleotides can be targeted to specific sequences (for a recent review see Seidman and Glazer, J Clin Invest 2003;112:487-94).

In general, the triplex-forming oligonucleotide has the sequence correspondence:

oligo	3'--A	G	G	T
duplex	5'--A	G	C	T
duplex	3'--T	C	G	A

However, it has been shown that the A-AT and G-GC triplets have the greatest triple helical stability (Reither and Jeltsch, BMC Biochem, 2002, Sept12, Epub). The same authors have demonstrated that TFOs designed according to the A-AT and G-GC rule do not form non-specific triplexes, indicating that the triplex formation is indeed sequence specific.

Thus, for any given sequence in the regulatory region a triplex forming sequence may be devised. Triplex-forming oligonucleotides preferably are at least 15, more preferably 25, still more preferably 30 or more nucleotides in length, up to 50 or 100 bp.

Transfection of cells (for example, via cationic liposomes) with TFOs, and formation of the triple helical structure with the target DNA induces steric and functional changes, blocking transcription initiation and elongation, allowing the introduction of desired sequence changes in the endogenous DNA and resulting in the specific downregulation of gene expression. Examples of such suppression of gene expression in cells treated with TFOs include knockout of episomal *supFG1* and endogenous *HPRT* genes in mammalian cells (Vasquez et al., Nucl Acids Res. 1999;27:1176-81, and Puri, et al, J Biol Chem, 2001;276:28991-98), and the sequence- and target specific down-regulation of expression of the Ets2 transcription factor, important in prostate cancer etiology (Carbone, et al, Nucl Acid Res. 2003;31:833-43), and the pro-inflammatory ICAM-1 gene (Besch et al, J Biol Chem, 2002;277:32473-79). In addition, Vuyisich and Beal have recently shown that sequence specific TFOs can bind to dsRNA, inhibiting activity of dsRNA-dependent enzymes such as RNA-dependent kinases (Vuyisich and Beal, Nuc. Acids Res 2000;28:2369-74).

Additionally, TFOs designed according to the abovementioned principles can induce directed mutagenesis capable of effecting DNA repair, thus providing both downregulation and upregulation of expression of endogenous genes (Seidman and Glazer, J Clin Invest 2003;112:487-94). Detailed description of the design, synthesis and administration of effective TFOs can be found in U.S. Patent Application Nos.

2003 017068 and 2003 0096980 to Froehler et al, and 2002 0128218 and 2002 0123476 to Emanuele et al, and U.S. Pat. No. 5,721,138 to Lawn.

Regardless of the methods described hereinabove, the present invention may also be effected by using mutant plants or plant variants, which do not accumulate these metabolites or compounds (1, 16). Such plants can be used for expressing and/or purifying the polypeptide of interest. Alternatively, such plants can be crossed with the transgenic plants expressing the polypeptide as described hereinabove. Next generations will include plants, which both express the polypeptide of interest and produce low levels of undesired compounds.

Plants generated or selected according to the above is preferably capable of accumulating less than 50 % of average volume of undesired compounds in the trichome cells of the pant species.

The present invention also envisages a method of producing a polypeptide of interest in plant trichomes. Such polypeptides can be endogenous to the trichome or exogenous polypeptides, which can be used as pharmaceuticals (e.g., antibodies, antigens, ligands, growth factors, enzymes, structural proteins), industrial proteins and enzymes, therapeutics for veterinary use, proteins for molecular laboratories and diagnostics, nutraceuticals or cosmeceuticals.

The method is effected by expressing the polypeptide in the plant trichomes, as described above, and down-regulating a level of at least one molecule endogenous to the plant trichomes wherein such a molecule is capable of interfering with the expression accumulation or stability of the polypeptide of interest.

Plants which may be utilized for trichome specific expression in accordance with the present invention, are preferably selected or generated capable of generating (i) a trichome size of at least 10 %, at least 20 %, at least 30 %, at least 40 %, at least 50 %, at least 80 % above average size of the plant species; (ii) leaf surface size at least 5 %, at least 10 %, at least 15 %, at least 25 %, at least 30 %, at least 40 % above average size of the plant species; and/or (iii) total leaf number at least 10 %, at least 20 %, at least 30 %, at least 40 %, at least 50 %, at least 80 % above average leaf number of the plant species; (iv) trichome density on the abaxial size of the leaf at least 10 %, at least 20 %, at least 30 %, at least 40 %, at least 50 %, at least 80 % above average trichome density of the plant species; (v) trichome density on the adaxial size of the leaf at least 100 % above average trichome density of the plant

species; (vi) shoot internode length of at least 5 %, at least 10 %, at least 15 %, at least 25 %, at least 30 %, at least 40 % above average length of the plant species; (vi) trichome density above 50,000 trichomes/gr leaf tissue; and/or (vii) trichome shape different than that of the plant species.

Plant architecture can be designed using genetic or non-genetic approaches [Weston (1989) J. Amer. Soc. Hort. Sci. 114:492-498; Antonious (2001) J. Environ. Sci. Health B. 36(6):835-48].

A number of genes which are associated with trichome development and morphogenesis were revealed through genetic studies. These genes may regulate trichome initiation, devision rate of trichome cells and/or trichome cell ploidy number. A mutation in the TTG gene (GenBank Accession Nos. TTG1 - AT5G24520 , TTG2 - AT2G37260) results in loss of leaf trichomes. Another example may be AGL16 (GenBank Accession No. NM_115583), a recently discovered MADS-box gene that is expressed in trichomes [Alvarez-Buylla (2000) Plant J. 24:457-466]. Yet another example is, KIC (GenBank Accession No. AY363866), a novel Ca^{2+} binding protein with one EF-hand motif, which interacts with a microtubule motor protein and regulates trichome morphogenesis [Reddy Plant Cell. (2004) 16:185-200]. Other genes, which affect trichome size and/or distribution, include but are not limited to the UPL family of genes (e.g., UPL3, GenBank Accession No. AY265959), STICHEL [GenBank Accession No. AF264023, Ilgenfritz et. al. (2003) Plant Physiol. 131:643-55], COT1 [Szymanski et.al. (1998) Genetics. 149:565-77], ZWICHEL (GenBank Accession No. AF002678, Oppenheimer et. al. (1997) Proc Natl Acad Sci U S A. 10;94:6261-6], GL1 (GenBank Accession No. AF263690), GL3 (GenBank Accession No. AT5G41315), GL2 (Acc. No. AT1G79840.1). It is conceivable that such genes when over-expressed may increase trichome size and/or distribution. For example, the GL-3 homologue R gene of maize causes trichome formation when over-expressed in Arabidopsis [Schellmann (2002) EMBO J. 21:5036-5046], indicating that such a manipulation is feasible. Overexpression of heterologous genes in plants is further detailed hereinbelow.

Non-transgenic approaches for modifying trichome size and/or distribution include chemical or physical mutagenesis [Szymanski et. al. (1998) Plant Cell. 10:2047-62], somaclonal variation [Saieed et.al. (1994) Tree Physiol. 14:17-26; Guo

et. al.(2003) Shi Yan Sheng Wu Xue Bao. 36:202-8] and induction of polyploidy [Melaragno et. al. (1993) Plant Cell. 5:1661-1668].

Trichome density can be increased by exposure to differentiating factors (i.e., non-genetic approcaches). For example, day length (16). Alternatively, physiological concentrations of ethylene have been shown to promote trichome formation [Kazama (2001) Plant Physiol. 117:375-83]. γ-radiation can be used to induce trichome formation [Negata (1999) Plant Physiol. 120:133-120].

Alternatively or additionally, trimming may be used to increase the number of leafs of the plant and as such increase the number of trichomes (see Example 7 of the Examples section).

Plants of the present invention are preferably sterile (i.e., having no viable pollen or seeds) to prevent spreading of genetic material to the surrounding environment. Sterilized plants can selected from mutant plants produced by for example chemical mutagenesis, physical mutagenesis or by somaclonal variation. Alternatively sterilized plants can be generated by silencing of fertility genes [Siaud et. al. (2004) EMBO J. 23:1392-401; Suzuki et. al. (2004) Plant J. 37:750-61; Li et. al. (2004) Plant Cell. 16:126-43; Krishnakumar and Oppenheimer(1999) Development. 126:3079-88].

Once plants are produced in accordance with the present invention, trichome content is purified to extract the molecules expressed therein or the products thereof.

Mechanical and chemical methods of isolating trichomes and trichomes exudates and content are known in the art. Such methods include the use of solvent containing microcapillary for dissolving the exudates. Measures are taken, though, to select a solvent which does not interfere with the activity or stability of molecules thus purified. Another method for removing trichomes include the use of forceps. A more efficient method for isolating exudates is by washing the surface with an organic solvent. Again, measures are taken to select a solvent which does not interfere with the activity or stability of molecules thus purified. Trcihomes may also be produced by brushing surfaces, shaking in an aqueous solution with an abrasive or freezing the tissue and then brushing [see McCaskill (1992) Planta 187:445-454; Wang (2001) Nature Biotechnology 19:371-374].

In order to facilitate collection of the trichome-produced molecule, the present inventors have devised a novel approach for large-scale collection of trichome

exudates and/or their content. This approach is simple to execute, does not require special technical skills, is cost effective and enables collection of large amounts of trichome exudates and content.

Trichome contect collection according to the present invention is effected by incubating a trichome containing plant tissue in a liquid (e.g., water) such that trichome exudates and content is released into the liquid. To avoid leaching of tissue components other than trichome, liquid incubation is effected while avoiding friction between the trichome containing plant tissue and a solid surface. Thereafter, the liquid is collected, thereby harvesting the trichome exudates and content.

For example, trichome containing plant tissues, such as, shoots, leaves and flowers collected from plants can be incubated for 30-60 seconds under agitation (60 times per minute) in water or any other liquid, which allows release of trichome content and exudates, while avoiding leaching of other tissue components. Preferably avoided are non-polar solvents, such as chloroform and hexane.

The liquid is preferably supplemented with antioxidants such as citric acid, ascorbic acid and sodium bisulfite, which reduce the activity of trichome components (e.g., PPO, see Example 5 of the Examples section interfering with purification of the molecules.

Further purification of the molecules can be effected using any chemical or biochemical method known in the art depending on the chemical nature of the molecule and its intended use. Such methods include, but are not limited to, chromatography methods such as thin layer, affinity, gel filtration and ion-exchange.

Collection of trichome exudates and content can be effected manually or by employing a collection apparatus specifically designed for such a purpose.

Thus, the present invention also envisages an apparatus for mechanical harvesting of trichomes and/or trichome exudates and content (illustrated in Figure 4a), which is referred to herein as apparatus **10**.

Apparatus **10** includes a collector **12** (e.g., brush, foreceps, arm) which is designed and configured for collecting trichomes and/or trichome exudates and content from a trichome containing plant tissue **14**. Accordingly, collector **12** includes a collecting mechanism **13** for holding plant tissue **14** and a fluid filled reservoir **16** including fluid **18** in which plant tissue **14** is agitated by collecting mechanism **13**.

Reservoir **16** also serves for storing collected trichomes and/or trichome exudates and content.

As is illustrated in Figures 4b-c, to enable agitation of plant material **14** within the fluid of reservoir **16**, apparatus **10** (collector **12**) includes a vibrating mechanism **20** which is fitted with a motor or servo and a power unit either to collecting mechanism **13** (Figure 4b) or to reservoir **16** (Figure 4c). Apparatus **10** may also include an actuating unit **22** and a timer **24** communicating with actuating unit **22**. Reservoir **16** may also include at least one liquid channel **26** and pump **28** for transferring the liquid with the trichomes out of the reservoir and into collection containers or directly to a chromatography device for further separation and molecule isolation.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531;

5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., Eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

EXAMPLE 1

Cloning promoter regions of trichome expressed genes and identifying trichome-active promoters

Promoters suitable for expressing proteins in trichomes were identified by sequencing the genomic DNA upstream region of various cDNAs obtained from genes expressed in leaf tissues.

MATERIALS AND METHODS

Isolation and cloning of trichome promoter sequences in a binary vector:

The NCBI database of 126,000 tomato expressed sequence tags (ESTs) (including 5,000 ESTs originated from cDNA libraries originated from the trichome tissues) and all transcribed nucleotide sequences described in literature or directly

submitted to NCBI (cDNAs) were used for the identification of trichome active promoters. Keywords representing each sequence and expression pattern thereof were collected and stored in a database.

LEADS™ software (Compugen, IL) was used for clustering and assembling the tomato sequences and provided more than 20,000 clusters representing different genes. An expression profile annotative summary was designed for each cluster by pooling all keywords of each sequence represented in the cluster. Clusters were selected based on trichome EST number and percentage out of total ESTs present in each cluster. Clusters were analyzed for ORFs using Vector NTI suite (InforMax, U.K.) version 6. ORFs of each gene were compared to Genbank database, using Blast (http://www.ncbi.nlm.nih.gov/BLAST/) and for the highest homologous ORF the position of the ATG start codon and stop codon was compared. Accordingly, most of the sequences described herein were predicted to posses the full length ORF. Clusters were classified as trichome-specific (i.e. more than 90% of ESTs in a cluster were originated from trichome cDNA libraries) or trichome expressed (i.e. at least one of the ESTs in a cluster was originated from trichome cDNA libraries).

RT-PCR - To verify the levels of expression and trichome specificity Reverse Transcription following quantitative (Real-Time) PCR (RTqPCR) was performed on total RNA extracted from either leaves, trichome cells or leaves minus trichome cells.

mRNA levels were measured for three genes, previously predicted bioinformatically to express to high levels and specifically in trichome cells.

Trichome cells were harvested from tomato mature leaves by first freezing the leaves, just above liquid nitrogen and then brushing both sides of the leaves with paint brush, previously chilled in liquid nitrogen. Total RNA was extracted from leaves, trichome cells or leaves minus trichome cells of tomato using Rneasy plant mini kit (Qiagen, Germany) using the protocol provided by the manufacturer. Reverse transcription was performed using 1.5 μg total RNA, using 300 U Super Script II Reverse Transcriptase enzyme (Invitrogen), 225ng random deoxynucleotide hexamers (Invitrogen), 500 μM dNTPs mix (Takara, Japan), 0.2 volume of x5 RT buffer (Invitrogen), 0.01M DTT, 60U RNAsin (Promega), DEPC treated DDW was added up to 37.5 μl.

RT reactions were incubated for 50 min at 42 °C, followed by 70 °C for 15 min. cDNA was diluted 1:20 in Tris EDTA, pH=8. 5mL of the diluted cDNA was used for qPCR.

To normalize the expression level between the different tissues specific primers were designed for the following housekeeping genes: Actin (SEQ ID NO: 72), GAPDH (SEQ ID NO: 73), and RPL19 (SEQ ID NO: 74). The following primers were used for qPCR: Actin F primer: CCACATGCCATTCTCCGTCT (SEQ ID NO: 77), R primer GCTTTTCTTTCACGTCCCTGA (SEQ ID NO: 78); GADPH F primer TTGTTGTGGGTGTCAACGAGA (SEQ ID NO: 79) , R primer ATGGCGTGGACAGTGGTCA (SEQ ID NO: 80); RPL19 F primer CACTCTGGATATGGTAAGCGTAAGG (SEQ ID NO: 81), R primer TTCTTGGACTCCCTGTACTTACGA (SEQ ID NO: 82); TR2 F primer tctcttcaattaggtacccgtcttg (SEQ ID NO: 83), R primer TGAATTTTGCCGTCATTGTCC (SEQ ID NO: 84); TR4 F primer GGGTTTAGACGTATCCGAAGGTC (SEQ ID NO: 85), R primer GCTCGTTTCCAATTTTCAGTAGAGA (SEQ ID NO: 86); TR5 F primer TTACGTGCCCAACTGAACACA (SEQ ID NO: 87), R primer CAATGCAATCAGCCCATGC (SEQ ID NO: 88).

qPCR was performed on cDNA (5 µL), using x1 iQ™ SYBR Green super mix (BioRad), forward and reverse primers 0.3 µM each, and DDW was added up to 20 µL.

qPCR reaction was performed in iCycler real-time PCR machine (BioRad) 95 °C for 3min, 40 times of 95 °C for 15 sec and 1min at 60 °C, followed by 95 °C for 15 sec, 60 °C for 60 sec, and 70 times of 60 °C for 10 sec +0.5 °C increase in each cycle.

The levels of expression (Qty) measured in the qPCR were calculated using the efficiency (E) of the amplification reaction and the corresponding C.T. (the cycle at which the samples crossed the threshold) $Qty=E^{-C.T.}$. This calculation method assumes that the efficiencies of the reactions of the GOI (gene of interest) and of the housekeeping genes are similar. In general the efficiencies of the reactions were 100% +/- 5%. .

Results are summarized in Figures 6a-d.

Figures 6a-d show that all three selected genes (i.e. TR2, TR4, and TR5) were expressed at high leves, up to 21 times higher than the housekeeping genes, in

trichome cells. In all cases expression was higher in trichomes compared to leaves, and in *L. hirsutum* compared to *L. esculentum* plants. Hence the promoter sequences, upstream to the gene sequences, were cloned from *L. hirsutum* gDNA.

In order to clone these promoter sequences and 5' untranslated region (5' UTR) upstream of the ATG starting codon, total genomic DNA was extracted from plant leaf tissues of 4 week old plants of the following species: cultivated tomato (*Lycopersicon esculentum*, var 870), wild tomato species (*Lycopersicon hirsutm,* var LA 1777 and *Lycopersicon pennellii*, var LA 716), tobacco (*Nicotiana tabaccum*, var NN) or cotton (*Gossypium hirsutum* var Acala 23). DNA extraction was effected using DNA extraction kit (Dneasy plant mini kit, Qiagen, Germany). Inverse PCR (IPCR), DNA digestion, self-ligation, and PCR reaction were performed on genomic DNA, following a well established protocol (http://www.pmci.unimelb.edu.au/core facilities/manual/mb390.asp) with the following modifications. To avoid mistakes in the IPCR, first the genomic sequence of the 5' sequence of a relevant cDNA (i.e. including introns) was identified to produce Genomic Island (GI). The desired region from the genomic DNA was PCR-amplified using direct oligonucleotide primers designed based on the cDNA cluster sequence, as was predicted by the Leads software (Compugen, IL). PCR reaction was performed in a DNA thermal cycler, using common PCR conditions (for example: 92 °C/3 min followed by 31 cycles × [94 °C/30 sec; 56 °C/30 sec; 72 °C/*3* min] followed by 72 °C/10 min). PCR products were purified using PCR purification kit (Qiagen) and sequencing of the amplified PCR products was performed, using ABI 377 sequencer (Amersham Biosciences Inc).

Primer sequences of each plant and the resultant GI sequence (i.e. the genomic sequence which was amplified using the primers) are listed in Table 1, below.

Table 1

ID/Plant	*Forward primer/SEQ ID NO:*	*Reverse primer/SEQ ID NO:*	*Product size/SEQ ID NO:*
TR2 (*L. hirsutum*)	atggaagtaactttgttgtatagtac/SEQ ID NO: 1	GCCAGTGATCACCATAAGGA G/SEQ ID NO: 2	376/SEQ ID NO: 3
TR4 (*L. hirsutum*)	Ttctttggttcttcaatgttgg/SEQ ID NO: 4	TTTGTAATGTCATTGGGAGGT C/SEQ ID NO:5	410 bp of 5' prime region out of about 3500 bp of amplified PCR product SEQ ID NO: 6; Note - 3500 bp were amplified by PCR, out of which only 5' prime 410 bp were sequenced
TR5 (*L. hirsutum*)	Gggtaatattcatttgattttcc/SEQ ID NO: 6	AACCTGCTTTACATGTTTCAA G/ SEQ ID NO: 7	431 bp/ SEQ ID NO: 9

To increase amplification efficiency as needed a different amplification technique [UP-PCR (20)] was employed. Briefly, UP-PCR technique was used in order to amplify unknown upstream region of a known cluster sequence. Generally, the procedure involved four oligonucleotide primers: two sequence specific primers (SPs, external and internal) (listed below), both having the same orientation of 3' end towards the unknown, yet desired, 5' region of the gene, and two universal walking primers (WP28 and sWP). Reaction mixtures were generated as follows: sample mixture (SM) - genomic DNA of appropriate plant (tomato or cotton) species (30-40ng), WP28 primers (20 pmol), and DDW was added to a final volume of 10 µl; Polymerase mixture (PM) – dNTPs (Roche, Switzerland) (10mM each), Expand Long Template Enzyme mix (Roche, Switzerland) (1U), 10 X buffer supplied with the enzyme and DDW was added to a final volume of 8 µl. SM was placed in a thermocycler (Biometra, USA), where it was subjected to an amplification program of 1 minute at 90 °C, held (pause) at 80 °C until PM was added, 30 seconds at 15 °C, 10

minutes at 25 °C, 3 minutes at 68 °C, held at 90 °C until the external SP (2 µl of 10 µM concentration) was added. The process was followed by external PCR reaction of 30 seconds at 92 °C, 10 seconds at 94 °C, 30 seconds at 65.5 °C, 3 minutes at 68 °C, for 30 cycles followed by final extension of 10 minutes at 68 °C.

External PCR products (diluted 5000 – 25000 fold) were used as template and subjected to amplification using specific internal sWP and SP (30 pmol each) primers, 1U Ex Taq (Takara), in 50µl reaction volume. Internal PCR reactions were subjected to an amplification program of 2 minutes at 92 °C, followed by 30 seconds at 94 °C, 30 seconds at 58 °C, and 3 minutes at 72 °C for 30 cycles and a final extension of 10 minutes at 72 °C. IPCR/Up-PCR products were purified (PCR Purification Kit, Qiagen, Germany) and sequenced (ABI 377 sequencer, Amersham Biosciences Inc). Table 2, below, lists primers and products of IPCR/Up-PCR reactions.

Table 2

ID/Plant	***Amplification method***	***External primers***		***Internal primers***		***Product SEQ ID NO:***
		sWP28/ SEQ ID NO:	***SP (external)/ SEQ ID NO:***	***sWP/ SEQ ID NO:***	***SP (internal)/ SEQ ID NO:***	
TR2/*L. hirsutum*	UP-PCR	TTTTTTTTT TTGTTTGT TGTGGGGG TGT/10	GGAAGTTT AAGTAGTG GGCTTG/11	TTTTTG TTTGTT GTGGG/ 12	GTGGGCTT GGTGGTAG ATTC/13	14
TR4/*L. hirsutum*	UP-PCR	TTTTTTTTT TTGTTTGT TGTGGGGG TGT/10	GTTGAGTC CACGAGCA GACAC/15	TTTTTG TTTGTT GTGGG/ 12	CGAGCAGAC ACTGTCAGA GG/16	17
TR5/*L. hirsutum*	UP-PCR	TTTTTTTTT TTGTTTGT TGTGGGGG TGT/10	ATTCACAA GGTTGTGG ATGAGG/18	TTTTTG TTTGTT GTGGG/ 12	GATGAGGT GTTTGGGT GCAC/19	20

For cloning the putative promoters and 5' UTRs, an additional PCR amplification was effected using a new set of primers (below) which included 8-12 bp extensions having one restriction site (*Hind*III, *Sal*I, *Xba*I, *Bam*HI, or *Sma*I) on the 5'

prime end thereof. For each promoter, restriction sites that do not exist in the promoter sequence were selected. Moreover, the restriction sites in the primer sequences were design such that the resultant PCR products were cloned into the binary vector pPI in the right orientation, upstream of the GUS reporter gene.

The plasmid pPI was constructed by inserting a synthetic poly-(A) signal sequence, originating from pGL3 basic plasmid vector (Promega, Acc No U47295; bp 4658-4811) into the *Hind*III restriction site of the binary vector pBI101.3 (Clontech, Acc. No. U12640).

Table 3, below lists the restriction enzymes containing primers, and the SEQ ID NO: of the resultant PCR products. Restriction sites within each primer are indicated by bold letters.

Table 3

ID/Plant	***Forward primer - Restriction enz./SEQ ID NO:***	***Reverse primer - Restriction enz./SEQ ID NO:***	***Product SEQ ID NO:***
TR2 (*L. hirsutum*)	(HindIII): 5'-AATTT**AAGCTT**GTGTCGCTC AGCCCCTACTC -3'/21	(SalI): 5'-AAATT**GTCGAC**ATCTCAAC TTGTTGCACTGAATTG -3'/22	23
TR4 (*L. hirsutum*)	(SalI): 5'-CCTA**GTCGAC**GGTGTTAAA TGGTGGGTTGG -3'/24	(BamHI): 5'-TT**GGATCC**GAGCAGACACT GTCAGAGG -3'/25	26
TR5 (*L. hirsutum*)	(HindIII): 5'-TTTCC**AAGCTT**GACCTGCTC TGATACCAATTG -3'/27	(BamHI): 5'-CC**GGATCC**TCGTAAGGAGT TTGTAATATG -3'/28	29

PCR products were purified (PCR Purification Kit, Qiagen, Germany) and digested with the restriction sites according to the primers used (Roche, Switzerland). The digested PCR products were re-purified and cloned into the binary vector pPI, which was digested with the same restriction enzymes. PCR product and the linearized plasmid vector were ligated using T4 DNA ligase enzyme (Roche, Switzerland).

EXAMPLE 2

Cloning of trichome active promoter sequences

Several genomic sequences were already described and validated in the literature as trichome-specific promoters. In most cases promoter validation was effected in tobacco plants. However, none of these sequences were analyzed in tomato plants. Hence there is no way to predict which of these promoters will be active in tomato.

MATERIALS, METHODS AND RESULTS

A previously described tobacco promoter, (8) was isolated from genomic DNA (gDNA) of *Nicotiana tabaccum*, var Samsun NN using two sets of overlapping primers: 1. Forward- 5'- AAA**TCTAGA**CTACCATCGCTAGTAATCGTG -3' (SEQ ID NO: 30) and Reverse- 5'- GTTGAAGAACTGCAT**CCCGGG**AGG -3' (SEQ ID NO: 31) to provide the sequence product set forth in SEQ ID NO: 32 (, TR25-2, SEQ ID NO: 67); 2. Forward- 5'- AAA**TCTAGA**TAAGTTGATAAAGCTAATTTCTC -3' (SEQ ID NO: 33) and Reverse- 5'- TTT**CCCGGG**ACCTGGAGGCAATC -3' (SEQ ID NO: 34) to provide the sequence product set forth in SEQ ID NO: 35 (TR25-3, SEQ ID NO: 68).

Primers sequences included additional restriction sites XbaI (Forward primers) and SmaI (Reverse primers), indicated in bold letters, to facilitate further cloning.

Each PCR product was digested with XbaI and SmaI (Roche) and introduced via ligation, using T4 DNA ligase (Roche), into pPI plasmid, digested with the same restriction enzymes.

A cotton promoter, previously described in (7), was isolated from gDNA of *Gossypium hirsutum*, var. Acala 23, and *Gossypium barbadense* var. Pima 15 using the primers: Forward- 5'- TAT**AAGCTT**TAAGTTTAAATCCTATTGTAGTG -3' (SEQ ID NO: 36) and Reverse- 5'- C**GGATCC**ATTAATCACAAGAAAAAC -3' (SEQ ID NO: 37) to provide a genomic amplified sequence of Acala as set forth in SEQ ID NO: 38 (27A) and a genomic amplified sequence of Pima as set forth in SEQ ID NO: 39 (27P).

Primer sequences included additional restriction sites HindIII (Forward primer) and BamHI (Reverse primer), indicated in bold letters, to facilitate further cloning. PCR products were digested with HindIII and BamHI (Roche) and

introduced via ligation, using T4 DNA ligase (Roche), into pPI plasmid, digested with the same restriction enzymes.

EXAMPLE 3

Cloning of tomato PPO promoters and signal peptide

Tomato polyphenol oxidase (PPO, GenBank Accession No: Z12833 for PPOA, GenBank Accession No. Z12836 for PPOD) is the major protein in type VI trichomes of tomato (5). Hence it was expected that the promoter region upstream the PPO gene will direct the expression of foreign genes to the trichome cells. PPO is encoded by closely related, seven members, gene family. A previous publication identified which of the gene family members are preferably expressed in the trichome cells (5).

The genomic sequence of the PPO gene family was published. Still, in most cases, promoter activity was not tested for the sequences upstream of the genes.

MATERIALS, METHODS AND RESULTS

The promoter sequence of PPOA and PPOD was cloned from wild tomato (*Lycopersicon pennellii*) and cultivated tomato (*Lycopersicon esculentum*), respectively. Cloning of the putative promoter region of PPOA was effected by amplifying the genomic sequence upstream of the coding region, using the primers: Forward- 5'- AAAATTTGGGA**TCTAGA**AGGTGAGG -3' (SEQ ID NO: 40) and Reverse- 5'- CT**GGATCC**TATTGCTAGCTTTGGATGAAG -3' (SEQ ID NO: 41). The resultant genomic DNA amplified thereby is set forth in SEQ ID NO: 42 (T8). Primer sequences include additional restriction sites XbaI (Forward primer) and BamHI (Reverse primer), indicated in bold, to facilitate further cloning.

The resultant PCR product was digested with XbaI and BamHI (Roche) and introduced via ligation, using T4 DNA ligase (Roche), into pPI plasmid, digested with the same restriction enzymes.

Cloning of the putative promoter region of PPOD was performed by amplifying the genomic sequence upstream of the coding region, using the primers: Forward- 5'- ATGGAA**AAGCTT**ATGGACAGACTAAAACAC -3' (SEQ ID NO: 43) and Reverse- 5'- CT**GGATCC**TGTTGCTAGCTTTTGAATGAAA -3' (SEQ ID NO: 44). The resultant genomic DNA amplified thereby is set forth in SEQ ID NO: 45 (T11).

Primer sequences included additional restriction sites HindIII (Forward primer) and BamHI (Reverse primer), indicated in bold, to facilitate further cloning.

PCR product was digested with HindIII and BamHI (Roche) and introduced via ligation, using T4 DNA ligase (Roche), into pPI plasmid, digested with the same restriction enzymes.

Vast accumulation of PPO in trichomes is largely governed by protein import and storage within the thylakoid lumen of plastids, such as chloroplasts and leucoplasts (5, 12, 28). Protein import into the lumen is directed by a signal peptide on the amino terminal of the immature polypeptide of PPO. The immature polypeptide is imported first into the plastid stroma where the primary part of the signal peptide is cleaved. Later on, the second part of the signal peptide is cleaved, while the polypeptide is crossing the thylakoid membrane and the mature polypeptide is entering into the thylakoid lumen (28).

Hence to facilitate the accumulation of foreign proteins in trichomes, protein import into plastids might be crucial.

To test protein import, the native signal peptide of either PPOA or PPOD was amplified together with the putative promoter of each gene. Amplified products were cloned into pPI and fused, in frame, to the GUS reporter gene. Amplification of PPOA promoter together with only the initial part of the signal peptide, which directs protein to the stroma was done using the following primers: Forward- 5'-AAAATTTGGGA**TCTAGA**AGGTGAGG -3'(SEQ ID NO: 46, XbaI restriction site is indicated in bold) and Reverse- 5'- ACATGAAACTTTGAATGCTTTG-3' (SEQ ID NO: 47). The genomic amplified sequence of PPOA is set forth in SEQ ID NO: 48.

PCR product was digested with XbaI (Roche) and introduced via ligation, using T4 DNA ligase (Roche), into pPI plasmid, digested with XbaI and SmaI restriction enzymes.

Amplification of PPOD promoter and signal peptide which directs protein to the stroma was effected using the following primers: Forward- 5'-ATGGAA**AAGCTT**ATGGACAGACTAAAACAC -3' (SEQ ID NO: 49) and Reverse- 5'-TT**CCCGGG**ACATGAAACTTTGAATGCTTTG -3' (SEQ ID NO: 50). Genomic amplified sequence of PPOD is set forth in SEQ ID NO: 51.

Primer sequences included additional restriction sites HindIII (Forward primer) and SmaI (Reverse primer) to facilitate further cloning.

PCR product was digested with HindIII and SmaI (Roche) and introduced via ligation, using T4 DNA ligase (Roche), into pPI plasmid, digested with the same restriction enzymes.

Amplification of PPOD promoter and signal peptide which directs protein to the lumen was done using the following primers: Forward- 5'-ATGGAA**AAGCTT**ATGGACAGACTAAAACAC -3' (SEQ ID NO: 52) and Reverse- 5'-AA**CCCGGG**AGCCGATGCAGCTAATGG -3' (SEQ ID NO: 53). The resultant genomic amplified sequence of PPOD is set forth in SEQ ID NO: 54.

Primer sequences included additional restriction sites HindIII (Forward primer) and SmaI (Reverse primer), indicated in bold, to facilitate further cloning.

PCR product was digested with HindIII and SmaI (Roche) and introduced via ligation, using T4 DNA ligase (Roche), into pPI plasmid, digested with the same restriction enzymes.

EXAMPLE 4

Expression of therapeutic proteins in trichome cells

MATERIALS AND METHODS

The potential of trichomes to accumulate human therapeutic proteins can be estimated by expressing human interferon β gene or human growth hormone gene in trichome cells.

MATERIALS AND METHODS

Cloning of human interferon β into a binary vector - The gene for human interferon β (INFB, GenBank Accession No. NM_002176) was amplified from human genomic DNA using the following primers: Forward: 5'-**GGG**ATGAGCTACAACTTGCTTGGAT-3' (SEQ ID NO: 55) and Reverse: 5'-CTAG**GAGCTC**TTCAGTTTCGGAG-3' (SEQ ID NO: 56, a SacI restriction site on the primer is indicated in bold). The resultant sequence of the INFB gene is set forth in SEQ ID NO: 57.

Analysis of the INFB sequence revealed a codon usage that is similar to the codon usage of the tomato (data not shown).

The PCR product (SEQ ID NO: 57) was digested using *Sac*I restriction endonuclease (Roche) and cloned into pPI binary vector digested with SmaI and SacI, hence replacing the GUS gene. The newly formed binary plasmid was designated pINFB. Sequence analysis of the INFB gene in pINFB revealed that the INFB sequence was cloned in the right orientation. Trichome promoters together or without a plastid transit peptides (summarized in Table 4 below) were further cloned upstream of the INFB gene in pINFB.

Cloning of human growth hormone into a binary vector - The mature polypeptide of the Human-growth hormone gene (HGH, GenBank Accession No: V00519) was produced synthetically using GeneArt service (http://www.geneart.de/). The sequence was adjusted according to the tomato codon usage, while avoiding, as much as possible, high GC content and low complexity of DNA sequences. An ATG was added as a first codon to the mature polypeptide enabling sufficient translation. The restriction sites of *Sma*I and *Sac*I were added to the gene at the 5' prime end and 3' prime end, respectively. The sequence of the HGH gene is set forth in SEQ ID NO: 58.

The gene clone was provided in a PCR script plasmid vector. The gene was digested out of the plasmid using *Sma*I and *Sac*I restriction endonucleas (Roche) and cloned into pPI binary vector, replacing the GUS gene. The newly formed binary plasmid was named pHGH. Sequence analysis revealed that the inserted HGH gene in pHGH was cloned in the right orientation. Trichome promoters with or without a plastid transit peptides (summarized in Table 4, below) were further cloned upstream of the HGH gene in pHGH.

Agrobacterium transformation of binary plasmids expressing heterologous genes - *Agrobacterium tumefaciens* (strains LBA4404) competent cells were transformed with 0.5 µl binary plasmid by electroporation, using a MicroPulser electroporator (Biorad, USA), 0.2 cm cuvettes (Biorad, USA) and EC-2 electroporation program (Biorad, USA). Cells were incubated in LB medium at 28 °C for 3 hours and plated on LB-agar plates supplemented with 50 mg/L kanamycin (Sigma, USA) and 250 mg/L streptomycin. Plates were incubated at 28 °C for 48 hours until *Agrobacterium* colonies grew. These colonies were subsequently used for tobacco or tomato plant transformation.

Plant transformation and cultivation – Table 4, below, summarizes the constructs which were introduced into tomato plants.

Table 4

Promoter	*species*	*Transit peptide*	*gene*	*binary*
35S	CaMV	No	GUS	pPI
TR2	*L. hirsutum*	No	GUS	pPI
TR4	*L. hirsutum*	No	GUS	pPI
TR5	*L. hirsutum*	No	GUS	pPI
TR8	*L. pennellii*	No	GUS	pPI
TR8	*L. pennellii*	Stroma	GUS	pPI
TR11	*L. esculentum*	No	GUS	pPI
TR11	*L. esculentum*	Stroma	GUS	pPI
TR11	*L. esculentum*	Lumen	GUS	pPI
TR25-2	*N. tabaccum*	No	GUS	pPI
TR25-3	*N. tabaccum*	No	GUS	pPI
TR27-A	*G. hirsutum*	No	GUS	pPI
TR27-P	*G.barbadense*	No	GUS	pPI
TR2	*L. hirsutum*	No	INFB	pINFB
TR4	*L. hirsutum*	No	INFB	pINFB
TR5	*L. hirsutum*	No	INFB	pINFB
TR8	*L. pennellii*	No	INFB	pINFB
TR8	*L. pennellii*	Stroma	INFB	pINFB
TR11	*L. esculentum*	No	INFB	pINFB
TR11	*L. esculentum*	Stroma	INFB	pINFB
TR11	*L. esculentum*	Lumen	INFB	pINFB
TR25-2	*N. tabaccum*	No	INFB	pINFB
TR25-3	*N. tabaccum*	No	INFB	pINFB
TR27-A	*G. hirsutum*	No	INFB	pINFB
TR27-P	*G.barbadense*	No	INFB	pINFB
TR2	*L. hirsutum*	No	HGH	pHGH
TR4	*L. hirsutum*	No	HGH	pHGH
TR5	*L. hirsutum*	No	HGH	pHGH
TR8	*L. pennellii*	No	HGH	pHGH
TR8	*L. pennellii*	Stroma	HGH	pHGH
TR11	*L. esculentum*	No	HGH	pHGH
TR11	*L. esculentum*	Stroma	HGH	pHGH
TR11	*L. esculentum*	Lumen	HGH	pHGH

TR25-2	*N. tabaccum*	No	HGH	pHGH
TR25-3	*N. tabaccum*	No	HGH	pHGH
TR27-A	*G. hirsutum*	No	HGH	pHGH
TR27-P	*G.barbadense*	No	HGH	pHGH

Tomato transformation - Tomato transformation was carried out according to Fillati *et al.* (19). Briefly, *Lycopersicon esculentum* cv. Micro- Tom cotyledons were used for *Agrobacterium* based plant transformation. The Micro -Tom seeds were surface sterilized for 10 min in a 3 % sodium hypochlorite solution. The seeds were washed with DDW three times and soaked for three hours in fresh DDW, then plated into 0.5 L container with Nitsch medium, containing MS salts, 3 % sucrose, Nitsch vitamins and 0.8 % plant agar (Duchefa, Netherland).. The PH was adjusted to 5.8 prior to autoclaving for 20 min at 121 °C. 50 seeds where plated on 0.5 L sterilized container containing the germination medium and left at 25 °C in culture room, 16/8 hrs light/dark cycles, under light intensity of (150 $\mu Em^{-2}S$).. Seedlings where grown for 8 days.

Agrobacterium tumefaciens strain LBA4404 carrying an intact vir region which can mediate the introduction of the T-DNA from the bacteria into plants. The binary vector plasmids, originated from pPI, were introduced into the strain LBA 4404 as described above.

For co cultivation, a single colony from freshly streak LB plate supplemented with 300 μg/ml streptomycin and 50 μg/ml kanamycin (Sigma) was used to inoculate 5 ml LB overnight shaking at 28 °C. The inoculated 5 ml where added to 45ml LB supplemented with 300 μg/ml streptomycin and 50 μg/ml kanamycin to additional overnight at the same conditions. The overnight culture where centrifuged for 3060 rpm for 10 minutes and rinsed with 50 ml MS medium.

2.5 ml of fine tobacco suspension culture where plated on petri dishes (100x25mm) containing 50 ml of KCMS murashige minimal organics medium supplemented with 0.2 μg/ml 2.4D, kinetin 0.1 μg/ml, thiamine hydrochloride 0.8 μg/ml, potassium acid phosphate 200 μg/ml, biotin 0.5 μg/ml, folic acid 0.5 μg/ml, casein hidrolysat 800 μg/ml and plant agar 0.8%, PH 5.8 (Duchefa, Netherland)..

Whatmann paper filter no.1 (Whatmann) was autoclaved and placed on top the of the feeder plates. Any air bubbles and remaining tobacco suspension media were

excluded. The plates were incubated for 24 hours under low light conditions (10 $\mu Em^{-2}S$).

8 days old cotyledons were cut at both ends on MS medium and plated for 24 hours on the tobacco suspension plates in the same conditions.

The cotyledons were immersed in 5 ml of the rinsed *Agrobacterium* cells diluted in 50 ml MS medium in sterile Petri dish. The concentration of the bacteria was ~$5X10^8$ cfu /ml. Following 10 minutes the cotyledons were blotted carefully to remove any excess of bacterial suspension. 20 cotyledons were placed on the feeder plates for 48 hr for co-incubation with the bacteria under the same conditions.

Cotyledons were then transferred to 2 Zeatin Ribozide (ZR) regeneration medium (Duchefa, Netherland), containing 400 μg/ ml carbenicillin or 150 Ticarcillin/ potassium clavulanate to inhibit growth of Agrobacterium and Kanamycin 100 μg/ml to select for transformed tomato cells.

The cotyledons were transferred to fresh regeneration media after 1 month supplemented with 1 ZR 200 carbenicillin and 100 μg/ml Kanamycin.

Shoots where excised when they were approximately 1 cm long and transferred to 0.5 L containers supplemented with rooting media containing MS medium 50 μg/ ml kanamycin, 100 μg/ ml carbenicillin disodium, 2 μg/ ml IBA (Duchefa, Netherland). After approximately ten days the rooted explants were transferred into soil, under 100% humidity. The humidity was reduced gradually for 24 hours. After 24 hr the plants were transferred to the greenhouse.

Testing expression of Foreign Proteins in Trichomes:

A. GUS staining - Gus staining of tomato and tobacco plants was effected as previously described (15). Briefly: Leaves were fixed in 90 % ice cold acetone for 15 - 20 minutes (on ice), followed by removal of acetone and a double tissue rinsing with the Working Solution [25mM Sodium Phosphate (Sigma, USA) buffer pH=7, Ferricyanide (Sigma, USA) 1.25mM, Ferrocyanide (Sigma, USA) 1.25mM, Triton X-100 (Sigma, USA) 0.25%, EDTA(BioLab) 0.25mM] for 15-20 minutes. Rinse solution was removed, replaced with Staining solution [Working solution with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-GlcA, Duchefa) solubilized in N,N-Dimethylformamide (BioLab) 1.5mg/ml and Dithiothreitol (DTT, Bio Lab) 100mM] and incubated in the dark (tubes wrapped with aluminum foil) over night at 37 °C. Distaining was effected by sinking the plant tissue in 70 % ethanol and heating

at 50 °C for ~ 2 hours. The distaining step was repeated until the plant tissue became transparent except the blue stained regions. Distained plants were stored in 70 % ethanol (BioLab) at room temperature.

ELISA - Human protein detection in plant tissues was effected using Human Interferon β ELISA Kit (R & D Systems) and Ultra-sensitive Human Growth Hormone ELISA Kit (Diagnostic Systems Laboratories, Inc), according to manufacture instructions.

Western Blot - Briefly, proteins extracted from the leaves were resolved on 12 % Tris-HCl Criterion gel (Bio-Rad Laboratories, Inc.) and transferred by electroporation onto PVDF membranes using the Bio-Rad Criterion Precast Gel System (100 V constant voltage at 4 °C, 1.5 hours). Pre-stained SDS-PAGE standards (Bio-Rad Laboratories, Inc.) were used as molecular weight markers. Primary antibodies were diluted 1:500 and the secondary antibody-HRP conjugate was diluted 1:15,000. The following anti- recombinant protein polyclonal antibodies were used: goat (Santa Cruz) or sheep (Biosource) polyclonal anti human IFN-β, goat polyclonal anti human GH (Santa Cruz), rabbit polyclonal anti *E.coli* β-glucuronidase (Molecular Probes). ECL and related reagents were obtained from Amersham Biosciences.

Bradford protein quantification test - Protein quantification was effected according to Bradford method [Bradford M., Analytical biochemistry, 72: 248 (1976)] using Bio-Rad Laboratories, Inc. reagent.

RESULTS

GUS staining was performed on leaves of T1 tomato and tobacco plants transfected with a binary vector including putative trichome specific promoters upstream of a GUS gene. Control plants included wild-type tomato and tomato transfected with GUS under the constitutive CaMV S35 promoter (Figures 1b-c). As is shown in Figures 1d-h under the regulation of TR2, TR5, TR11, TR25 and TR27 promoters GUS was expressed in a trichome-gland specific manner (Figures 1d-h). A light blue color was also found in the stalk cell immediately attached to the gland and in non glandular tissues. Results for tomato T1 generation are summarized in Table 6, below.

Table 6

Promoter	*Signal peptide*	*No of Independent T1 plants*	*Avg grade*	*Range*
35S	No	9	2.1	0-5
TR2	No	2	1.5	0-3
TR5	No	6	0.2	0-1
TR11	No	3	0	0
TR11	Stroma	4	0	0
TR11	Lumen	4	0.5	0-1
TR25-2	No	8	0	0
TR25-3	No	18	0.1	0-1
TR27-P	No	5	0.4	0-2

The results presented in Table 6 above indicate that TR2 and TR27-P are most effective in facilitating expression of heterologous genes in the tomato trichome. These results are of special significance since TR2 has never been described before as a trichome active promoter. Furthermore, the addition of a luman directing signal peptide seems to have facilitated expression in the trichome.

EXAMPLE 5

Novel methods for mechanical harvesting of proteins from trichomes

MATERIALS AND METHODS

Harvesting of Trichome proteins:

Four harvesting protocols were attempted to optimize protein purification.

Protocol 1 - Total trichome protein harvesting was performed by wiping fully expanded leaves with cotton swabs moistened with a solution of 200 mM Dithiothreitol (DTT, BioLab). Approximately 5-7 leaves were wiped per swab, with a total of about 1500 leaves. The crude trichome extract was squeezed from the swab using a syringe, and centrifuged (15000 x g for 15 minutes at 4 °C). To adsorb phenolic compounds, the supernatant (approximately 70 ml) was treated with Polyvinylpolypyrrolidone (PVPP; Sigma, USA) for 10 minutes on a gentle stirrer followed by centrifugation (15000g for 20 minutes) to remove the PVPP. Prior to application, PVPP was boiled in 10 % HCl for 10 minutes, washed extensively with DDW, air-dried for storage and soaked for at least 3 hours in 200 mM DTT solution

at 4 °C (ratios were 8-12 g PVPP / 250ml of 200mM DTT, 1-1.5g / 60-85ml extract). Extracts were concentrated in two steps of ammonium sulfate precipitation (20 %, 75 % of saturation). Solid ammonium sulfate (0%→20%: 114g/1L, 20%→75%: 382g/1L) was added to the extract until the desired concentration was reached while constant stirring. Thereafter the solution was held on ice for about 1 hour with occasional stirring. The precipitate was collected by centrifugation (15000 X g for 20 minutes at 4 °C) and dissolved in 50 mM Tris-HCl, pH 7 and 30mM NaCl.

Protocol 2 - Trichome cells and exudates were harvested directly into DDW which contained chemicals with antioxidant activity (i.e., citric acid, ascorbic acid or sodium bisulfite). Tomato leaves and shoots were soaked in a container. Gentle shaking of the tissue in the liquid medium caused the explosion of the glandular trichome cells, type VI and VII (see Figure 1a) and released the trichome exudates into the media. To eliminate the loss of significant amounts of liquid, tissues were lifted out of the medium and partially dried by shaking it vigorously, letting drops fall back to the container. Trichome yield was measured by inspecting the treated leaves. Trichome harvesting efficiency was calculated as the percentage of broken and exploded trichomes, out of total trichomes on a given leaf area.

Protocol 3 - Trichome cells and exudates were harvested directly into a liquid media. Tomato leaves or shoots were put in a container, filled with tap water. The container was closed and centrifuged at 20 rpm for 5 minutes. To eliminate the loss of significant amounts of liquid, tissues were lifted out of the medium and partially dried by shaking it vigorously, letting drops fall back to the container. Trichome yield was measured by inspecting the treated leaves. Trichome harvesting efficiency was calculated by percentage of broken and exploded trichomes, out of total trichomes on a given leaf area.

Protocol 4 - Trichome cells and exudates were harvested directly into a liquid media. Tomato leaves or shoots were put in a container, filled with tap water. The container was closed and water was poured on top of the leaves using pump which circulated the water in the container. Trichome yield was measured by inspecting the treated leaves. Trichome harvesting efficiency was calculated by percentage of broken and exploded trichomes, out of total trichomes on a given leaf area.

RESULTS

Protein harvest, purification, yield – Protocol 1 was effected on a commercial tomato variety, grown in a commercial greenhouse, which was designed and used for tomato fruit harvest. Tomato (*L. esculentum* var 591) plants were grown for 3 months. Plant architecture was designed by leaving two main shoots for each plant. Using Bradford analysis it was possible to calculate total protein yield. Harvesting about 1,100 leaves yielded 16 mg total protein (0.1 mg/ml). Total protein yield was resolved on Nu-PAGE Novex Bis-Tris gel, 12 % (Invitrogen) and protein bands were visualized by coomassie staining (Figure 2). A band harboring of an estimated size of 60 kDa was predicted to be the mature PPO protein according to previous reports (12, 29). PPO is estimated to count for 60 % of total proteins in the trichome.

It will be appreciated that although protocol 1 for protein harvesting is highly effective in collecting most of the trichome exudates, it is labor intensive and slow. The other described methods (2, 3, and 4) were tested to replace the mechanical harvesting step in method 1.

Tomato shoots (1 m long) were dipped in a container and were subjected to trichome mechanical harvesting using method 2, 3 or 4.

Results, showing the efficiency of each method are presented in the Table 7, below.

Table 7

Method	*Number of Shoots*	*Average Efficiency*	*Range Efficiency*
2	3	98	96-100
3	3	11.3	8-14
4	3	42	38-46

These results suggest that protocol 2 is the most effective for protein harvesting from trichomes.

High activity of PPO in trichome may affect protein harvesting and purification from trichomes. Thus, identification of chemicals which are strong antioxidants, non toxic, cheap and not affecting protein stability is mostly desired.

Three compounds were tested: citric acid, ascorbic acid and sodium bisulfite. Different concentrations of chemicals were used to identify the chemical with the highest antioxidant activity. Tomato (*L. esculentum*) young leaves (about 100mg)

were grinded at 4 °C in 200 µl of 10 mM Tris-HCl pH=8 buffer containing appropriate concentration of antioxidant (0.01 - 2.0 %w/v), incubated for about 2 hrs at 4°C and centrifuged at 14,000 rpm for 3 min in order to separate leaves-debris and liquid fraction. Decreasing of PPO activity was inspected by eliminating the production of brown color of the supernatant as a result of the oxidation of polyphenols. Table 8 below summarizes the minimal concentration that enables a decrease of 95% of PPO activity.

Table 8

chemical	*Efficient concentration*
Citric acid	1.0%
Ascorbic acid	1.0%*
Sodium bisulfite	0.05%

* - After overnight incubation at 4 °C all the samples undergone browning regardless of the ascorbic acid concentration.

Figure 3 shows browning of medium as a result of PPO activity, under different concentrations of Sodium bisulfite.

A buffer without any antioxidant was used as a negative control. DDT was used as a positive control at the concentration of 200 mM, which is known to eliminate PPO activity when harvested from trichome cells (12, 29).

EXAMPLE 6

An automated machine for trichome cells and trichome exudate harvesting

A machine was designed, to enable the automation and up-scaling of protein harvesting according to protocol 2. The purpose of the machine is to harvest trichome cells and exudates from full-grown, 3 month old tomato plants (about 1 meter long and 80 cm in diameter). The machine is built of 2 main parts, (i) 2 m high and 1 m in diameter of cylinder shaped container (either made from stainless-steel, glass or a plastic); and (ii) a 2.5 m arm which operates using a 2 speed engine.

The machine has four steps of operation:

1. The plant is tight to the arm manually. The arm is introducing the plant into the container, half full with the liquid medium.

2. The arm is slowly moving up and down (engine is operating on a slow speed) the plant in the liquid medium such that trichome cells and exudates are released into the medium, without damaging other tissues of the plant.

3. The arm is moving the plant just above of the liquid medium.

4. The arm is vigorously shaking the plant up and down (engine is operating on a high speed). Doing that most of the liquid, which remained attached to the plant tissues, is released and drops falls back to the container. The semi-dried plant is removed and a new plant is tight to the arm.

EXAMPLE 7

Determining plant architecture for optimizing trichome production

Typically, the architecture of cultivated tomato plants is designed via breeding to provide the highest fruit yields, in a given space, in a given time. Moreover hand labor is routinely being practiced to optimize plant architecture for that purpose. For trichome optimized expression, plant architecture needs re-design to optimize trichome production, in a given greenhouse space in a given time. Two approaches for increasing protein yield were employed essentially, increasing the number of trichomes in leaves; and increasing the number of leaves on the plant.

MATERIALS METHODS AND RESULTS

Increasing number of trichomes on leaves - Over 300 tomato cultivars were screened for trichome density. Leaves of 4 weeks old plants were inspected, and average trichome density was measured. Best performing cultivars were grown and trichome density was tested again on mature, 14 week old plants. Trichome density was compared to previously measured density of several tomato lines (1). The seven best performing cultivars were grown in the next season and trichome density was measured again, to check the heredity of trichome density of two generations. Table 9, below summerizes trichome density of the best performing cultivars.

Table 9

Var	*No. of Plants 1st generation*	*Avg. trichome number of 1st generation*	*No of Plants 2nd generation*	*Avg. trichome number of 2nd generation*
309_2	3	6	3	5.3
305	3	4.7	3	5.3
249_1	3	4.3	3	5.3
247	3	3.7	3	6
273_1	3	5.4	3	4.7

294	3	4.5	3	3.2
289	3	4.2	3	3

(Note – the number in the above table represent only the best performing cultivars, out of 300 tested).

Folding a single leaflet and inspecting the edge of the folded leaflet was performed in order to count trichome cells. Trichome number is all trichomes found on the edge of the leaflet under X 120 magnification using binocular microscope (Optika, Italy). Previous publication has calculated for *Lycopersicon hirsutum, var Glabratum* (Cultivar No 273_1 in this experiment) over 100,000 trichomes per 1 gram of leaf. Assuming trichome density in this experiment remains the same, best performing *L. esculentum* cultivars (No 309_2, 305, 249_1, 247, 294, 289), identified here, have the trichome number in the same order. Each leaflet was inspected 3-5 times and an average number was calculated for the leaflet. Three different leaflet from three different plants were inspected for each cultivar in each generation.

Four cultivars were identified with the highest density of type VI (Figure 1a) glandular trichomes on the upper part of the leaves. Among the five best performing cultivars, one belongs to *Lycopersicon hirsutum, var Glabratum.* (273_1) and the rest for cultivated tomato (No 309_2, 305, 249_1, 247,). Interestingly, the *Lycopersicon esculentum* species cultivars exhibited up to 20 fold more coverage of type VI trichomes compared to other cultivars within this species (not shown). Overall best cultivated cultivars possessed the same density of type VI trichomes, compared to the wild species *Lycopersicon hirsutum,* var *glabratum*, which is recognized as the highest trichome density in all *Lycopersicon* genus (1, 16).

Approach B - Increasing number of leaves in a plant - Tomato plant architecture was designed manually. 35 days old plants were planted in a greenhouse. Different mechanical treatments were applied to shape plant architecture during plant growth. To avoid the collapse of the plant bush, shoots were hanged from the greenhouse ceiling using plastic strings. The three best performing treatments for plant architecture, aiming to increase trichome yield by increasing the number of leaves produced are presented hereinbelow:

1. Plant shoot number is not limited, plant height is limited to 1m, flowers were cut-off before fruit set.

2. Plant shoot number is not limited, plant height is limited to 2m, flowers were cut-off before fruit set.

3. Plant shoot number is not limited, leading apical meristem was cut (i.e. breaking apical dominance) when reached 0.5m, flowers were cut-off before fruit set.

4. Plant shoot number was limited to two. Flowers and fruits remained untouched (A control treatment, usually applied for greenhouse tomatoes, grown for fruit set).

Three different indeterminant (i.e. the greenhouse type) tomato cultivars (namely 678, 1312, and 2545) previously identified as having high trichome density, were grown in the greenhouse for two months. The four above treatments were applied to five plants from each cultivar. Leaf number of each plant was calculated. Table 10, below summarizes the leaf number of tomato plants growing under different mechanical plant design .

Table 10

Var	*treat*	*N Rows*	*Mean(No)*	*Std Err(No)*
678	1	5	94.2	7.61
678	2	5	87.4	5.22
678	3	5	71	5.03
678	4	5	27.8	2.75
1312	1	5	125	24.56
1312	2	5	148.4	13.12
1312	3	5	123	10.22
1312	4	5	54.6	2.84
2545	1	5	95.6	23.89
2545	2	5	81.4	3.91
2545	3	5	81.8	12.92
2545	4	5	34.4	4.48

As is evident from Table 10, above and Figures 5a-c, a significant increase [at 0.05 level for cultivar 2545 (Figure 5c) and 0.01 level for cultivars 678 and 1312 (Figures 5a-b, respectively)] was observed in response to treatments number 1, 2, 3 compared to control (treatment No 4). Overall a 50 % increase in leaf number (2.25 to 3.39) was observed over the control. Trichome density and PPO enzyme activity in trichomes were measured in each plant to verify that the increase in leaf number is not correlated with a decrease in trichome or protein production. No significant change (at 0.05 level) was observed for either trichome number or protein accumulation, following growth in leaf number (data not shown).

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

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